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Genetics

Ecdysone-inducible gene expression in mammalian cells and transgenic mice

(retinoid X receptor/tetracycline/promoter/cre recombinase)

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ABSTRACT **During metamorphosis of *Drosophila melanogaster*, a cascade of morphological changes is triggered by the steroid hormone 20-OH ecdysone via the ecdysone receptor, a member of the nuclear receptor superfamily. In this report, we have transferred insect hormone responsiveness to mammalian cells by the stable expression of a modified ecdysone receptor that regulates an optimized ecdysone responsive promoter. Inductions reaching 4 orders of magnitude have been achieved upon treatment with hormone. Transgenic mice expressing the modified ecdysone receptor can activate an integrated ecdysone responsive promoter upon administration of hormone. A comparison of tetracyline-based and ecdysone-based inducible systems reveals the ecdysone regulatory system exhibits lower basal activity and higher inducibility. Since ecdysone administration has no apparent effect on mammals, its use for regulating genes should be excellent for transient inducible expression of any gene in transgenic mice and for gene therapy.**

Precise control of gene expression is an invaluable tool in studying development and other physiological processes. Other applications for regulated gene expression include inducible gene targeting, overexpression of toxic and teratogenic genes, antisense RNA expression, and gene therapy (1). For cultured cells, glucocorticoids and other steroids are commonly used to induce expression of a desired gene. In the past several years, a tetracycline-regulated system has been devised in which gene activity is induced in the absence of the antibiotic and is repressed in its presence (2–5). Recently, the tetracycline-based system has been utilized in transgenic mice (4, 5). Disadvantages to this system include the continuous treatment of tetracycline to repress expression and the slow clearance of antibiotic from bone, which interferes with quick and precise inductions. While this system has been improved by the recent identification of a mutant tetracycline repressor, which acts conversely as an inducible activator, the pharmacokinetics of tetracycline may hinder its use during development when a precise and efficient on–off switch is essential (6).

As an alternative to tetracycline, we have explored the use of the insect molting hormone ecdysone as a potential inducer. A pulse of the steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing genomic effects such as chromosomal puffing within minutes of hormone addition (7–9). Mediating this response is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) (10, 11). Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or the synthetic analog muristerone A. Unlike tetracycline-based strategies, transferring ecdysone responsiveness to mammalian cells and transgenic mice takes advantage of a naturally evolved steroid-inducible system. Advantages for ecdysteroid use include the lipophilic nature of the compounds for efficient penetrance into all tissues including the brain, short half-lives which allow for precise and potent inductions, and favorable pharmacokinetics that prevent storage and expedite clearance. Because ecdysteroids are neither toxic, teratogenic, nor known to affect mammalian physiology, they would appear to be ideal candidates for use as inducers in cultured cells and transgenic mice (D.N., unpublished data). Here we report the development and properties of a regulatory system which reveals ecdysone to be an efficient and potent inducer of gene expression in cultured mammalian cells and transgenic mice.

MATERIALS AND METHODS

Plasmids. CMX-EcR, CMX-USP, CMX-FXR, CMX-hRXRα, EcREx5-ΔMTV-Luc, CMX-GEcR, MMTV-Luc, and CMX-GR have been described (10, 12). CMX-VpEcR was constructed by ligation of an *Eco*RI fragment of psk-EcR and CMX-Vp16. CMX-VgEcR was generated by site-directed mutagenesis of CMX-VpEcR using the transformer mutagenesis kit (Clontech) and the mutagenic oligonucleotide 5′-TACAACGCCCTCACCTGTGGATCCTGCAAGG-TGTTTCTTTCGACGCAGC-3′. Mutagenesis of VpEcR to VgEcR altered the P box region of the DNA binding domain of EcR to resemble that of the glucocorticoid receptor (GR) (13). The following amino acids were altered: E282G, G283S, and G286V. EcREx4-ΔHSP-βgal was constructed by oligomerizing two annealed oligonucleotides containing the HSP-EcRE (10). EcREx4-Sp1x3-ΔHSP-βgal was made by ligating the following annealed oligonucleotides into the Asp718 site of EcREx4-ΔHSP-β-gal: 5′-GTACTCCCGGGGCGGGGC-TATGCGGGGCGGGGCTAATCGCTAGGGGCGGG-GCA-3′ and 5′-GTACTGCCCCGCCCCTAGCGATTAGC-CCCGCCCCGCATAGCCCCGCCCCGGGA-3′. ΔHSP is a minimal promoter derived from the *Drosophila* heat shock promoter with its enhancers deleted. To generate E/GREx4-ΔMTV-Luc, the oligonucleotides 5′-AGCTCGATGGACAAGTGCAT-TGTTCTTTGCTGAA-3′and5′-AGCTTTCAGCAAGAGAA-CAATGCACTTGTCCATCG-3′ were annealed, multimerized, and ligated into the *Hin*dIII site of ΔMTV-Luc. The resulting reporter contained four copies of the E/GRE. A *Bgl* II/(*Xho* I) fragment containing EcREx4-Sp1x3-ΔHSP-βgal was subcloned into *Bgl* II/(*Not* I)-digested pRC-CMV (Invitrogen), which contains a neomycin-resistance gene, resulting in construction of pRC-ESHβ. TETREx7-tk-luc, CMV-tTA-VP16, and CMV-rtTA-VP16 were obtained from H. Bujard (2–4).

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Abbreviations: EcR, ecdysone receptor; GR, glucocorticoid receptor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-gal, β-galactosidase; RXR, retinoid X receptor; EcRE, ecdysone response element; FXR, farsenoid X receptor; GRE, glucocorticoid response element.
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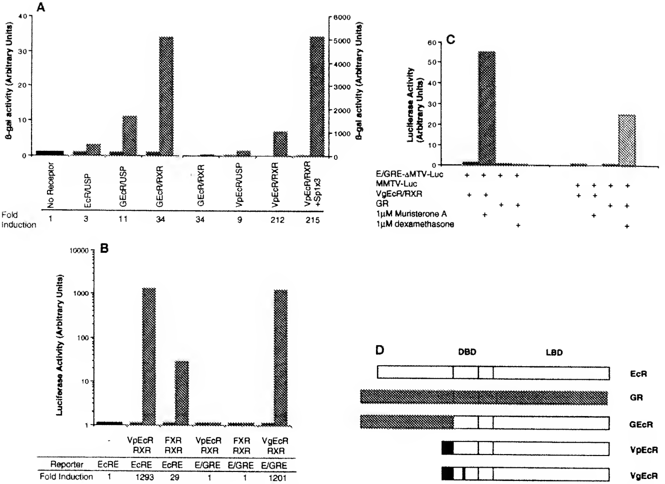

FIG. 1. (*A*) Optimization of ecdysone responsiveness using different modified EcR combinations. After transfection, cells were treated with either ethanol or 1 μM muristerone A. Numerical values on both sides of the figure are on the same scale, and the GEcR/RXR value was repeated for clarity. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A, respectively. (*B*) FXR and VpEcR activity on a EcRE and E/GRE responsive reporter. VpEcR, VgEcR, and the transfection without receptors were treated with 1 μM muristerone. FXR transfections were treated with 50 μM juvenile hormone III. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A/50 μM juvenile hormone III, respectively. (*C*) E/GRE and GRE are nonoverlapping response elements. Solid and hatched bars represent reporter activity with no hormone or 1 μM muristerone A/1 μM dexamethasone, respectively. (*D*) Schematic diagram of modified EcR derivatives. GEcR is a chimeric receptor containing the N-terminal transactivation domain of GR and the DNA and ligand binding domains of EcR. VpEcR is an N-terminal truncation of EcR fused to the activation domain of Vp16. VgEcR is identical to VpEcR except for the following point mutations in the P box of the DNA binding domain: E282G, G283S, and G286V. DBD, DNA binding domain; LBD, ligand binding domain.

Cell Culture and Transient Transfections. CV-1 cells were maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum (HyClone). Transient transfections were performed using Dotap transfection reagent (Boehringer

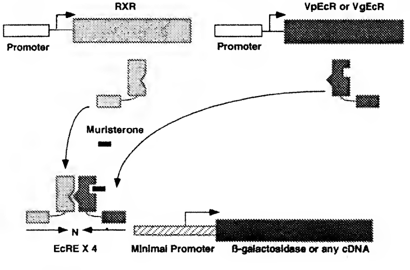

FIG. 2. Schematic diagram of ecdysone-inducible gene expression system. After expression of RXR and VpEcR, the two receptors can heterodimerize and transactivate the EcRE-containing promoter in the presence of hormone. The EcREs are placed upstream of a minimal promoter, which can drive the expression of any cDNA. For animals, two transgenic lines must be generated. The first line will express RXR and VpEcR. Tissue specificity will be conferred by selection of promoters that will then direct expression of the receptors. A second line will carry an ecdysone-responsive promoter controlling expression of a cDNA. Breeding of the two lines and treatment with muristerone will allow for temporal, dosage, and spatial specificity of cDNA expression.

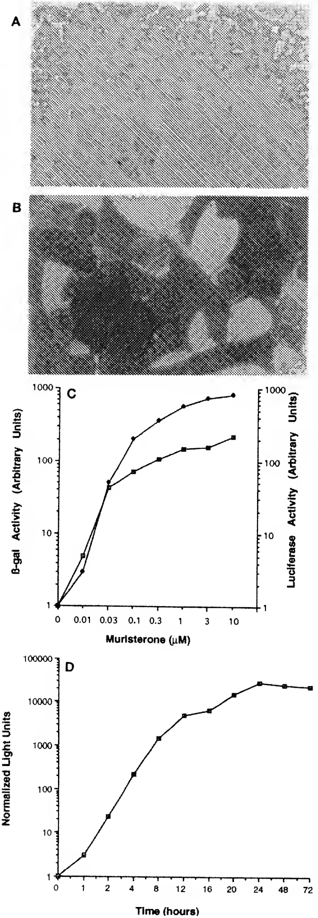

FIG. 3. (*A* and *B*) X-Gal staining of stable cell line N13. Of 14 G418-resistant clones, 10 demonstrated muristerone responsiveness. One of these cell lines, N13, was grown with (*A*) or without (*B*) the presence of 1 μM muristerone for 20 hr. The cells were then fixed and treated with X-Gal for 2 hr. (*C*) Dose-dependent activation of N13 cells with muristerone. N13 cells were grown with varying concentrations of muristerone for 36 hr and then assayed for β-gal activity (open squares) by standard *O*-nitrophenyl β-galactoside assay or for luciferase activity (solid circles). (*D*) Time course of luciferase activity of N13 cells treated with hormone. N13 cells were grown in separate wells in the presence of 1 μM muristerone, harvested at varying times, and assayed for luciferase activity.

Mannheim). Transfections using β-gal as the reporter were assayed either by Galactolight luminescent assay (Tropix) or by standard liquid *O*-nitrophenyl β-galactoside assay (ONPG) (Sigma). The values were normalized by cotransfection of CMX-luciferase. Transfections using luciferase as the reporter were assayed by standard techniques using luciferin and ATP. These values were normalized by cotransfection of CMX-β-gal. Hormone-treated cells were treated with ethanol, 50 μM juvenile hormone III (Sigma), 1 μM muristerone A (Zambon), or 1 μM dexamethasone (Sigma) unless otherwise noted. For comparison with the tetracycline repressor/activator, equimolar amounts of both reporter and activator plasmids were transfected. All transfections using doxycycline (Sigma) were performed in the dark as described (4).

Stable Cell Line Production. 293 cells were transfected with the linearized plasmids pRC-ESHβ allowed to recover 1 day prior to selection with G418 (1 mg/ml) (GIBCO). After 14 days of selection, 14 individual clones were isolated and grown separately in the presence of G418 (0.5 mg/ml). Of these, 10 exhibited muristerone responsiveness to differing degrees. Cell lysates were then assayed for β-gal and luciferase activities as already described. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining was performed on the stable cell lines. Cells were fixed briefly with 10% formaldehyde in PBS and then stained with X-Gal (Molecular Probes) for 2–6 hr.

Muristerone Treatment of Mice. All mice were treated with approved protocols of the Salk Institute for Biological Studies. For toxicity studies, adult mice were injected intraperitoneally with 20 mg of muristerone A suspended in sesame oil. The mice were then observed for ∼2 months. For teratogenic studies, pregnant mice were injected with 20 mg of muristerone A suspended in sesame oil and both the mother and pups were observed for 3 months. For muristerone bioavailability studies, adult mice were injected intraperitoneally with sesame oil with or without 10 mg of muristerone. After 12 hr, blood was drawn from the mice, and the serum was isolated by brief centrifugation of the whole blood. Serum from sesame oil injected mice was divided, and half was supplemented with muristerone to a final concentration of 10 μM. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRα, and EcREx5-ΔMTV-Luc.

Transgenic Mice. The following DNA constructs were prepared and subsequently injected into fertilized eggs: CD3-VpEcR, CD3-RXR, and ESHβ (14). CD3-VpEcR and CD3-RXR were mixed and coinjected while ESHβ was injected alone. Primary genotyping was performed by Southern blot analysis and transmission of transgenic mice was monitored by dot blot analysis. Expression of the receptors was determined by Northern blot analysis of RNA isolated from the thymus as well as other tissues for negative controls. Receptor-expressing mice were bred with reporter mice (containing ESHβ) to produce double-transgenic receptor/reporter mice. Double-transgenic lines were treated with muristerone A as described above and RNA was isolated 48 hr after hormone treatment. For Northern blot analysis, 15 μg of total RNA from various tissues including the thymus was run on a denaturing gel and

blotted onto a nitrocellulose membrane. The blot was probed with a radiolabeled β-gal-specific probe and exposed on film for 2 days.

RESULTS

Optimization of EcR Derivatives. Previous studies have shown that mammalian cells cotransfected with EcR and USP produce a 3-fold induction upon treatment with 1 μM muristerone, an ecdysone analog (10, 11) (Fig. 1*A*). To maximize the sensitivity of an ecdysone-inducible system, modifications of EcR were made. The N-terminal transactivation domain of EcR was replaced by the corresponding domain of GR (Fig. 1*D*). This new hybrid receptor named GEcR boosted muristerone responsiveness from 3- to 11-fold in a transient transfection assay (Fig. 1*A*). Replacement of EcR's natural heterodimeric partner, USP, by its mammalian homologue, the retinoid X receptor (RXR), produced a more potent ligand-dependent heterodimer, producing a 34-fold induction (Fig. 1*A*). A most potent heterodimer, however, was a combination of RXR and VpEcR, an N-terminal truncation of EcR attached to the VP16 activation domain resulting in a 212-fold induction (Fig. 1 *A* and *D*). Different from most nuclear receptor/VP16 fusion proteins, which exhibit high constitutive activity, VpEcR generates ligand-dependent superinduction while maintaining a very low basal activity (15, 16). In addition, the reporter vector was also modified by inserting consensus binding sites for the ubiquitous transcription factor Sp1 between the minimal promoter and the ecdysone response elements (EcREs). Sp1 has been shown to boost transcriptional activity of many promoters (17–19). The addition of Sp1 sites to the ecdysone responsive promoter increases the absolute activity 5-fold (Fig. 1*A*).

Construction of a Novel Ecdysone-Specific Response Element. Although no mammalian transcription factors have been shown to have a natural enhancer element like the EcRE, which is composed of two inverted half-sites of the sequence AGGTCA spaced by 1 nucleotide, it is difficult to preclude such a possibility. The recently cloned farsenoid X receptor (FXR) can very weakly activate certain synthetic promoters containing an EcRE in response to extremely high concentrations of farsenoids (14). For FXR-containing cells and transgenic mice, it would be undesirable if endogenous receptors can activate an EcRE-containing promoter. To circumvent this potential problem, the DNA binding specificity of VpEcR was altered to mimic that of GR, which binds as a homodimer to an inverted repeat of the sequence AGAACA spaced by 3 nucleotides. This altered binding specificity was achieved by mutating 3 amino acid residues of VpEcR in the P box of the DNA binding domain, a region previously shown to be essential for DNA sequence recognition (13). This hybrid receptor is named VgEcR and is responsive to a hybrid responsive element called the E/GRE, which contains two different half-sites, AGGTCA and AGAACA, spaced by 1 nucleotide (Fig. 1*B*). This response element is a hybrid between the glucocorticoid response element (GRE) and that of type II nuclear receptors like RXR, EcR, retinoic acid receptor, thyroid hormone receptor, etc. Although FXR can activate a promoter containing the wild type EcRE, it cannot activate one containing the E/GRE (Fig. 1*B*; note logarithmic scale). The E/GRE reporter is not activated by GR nor does VgEcR activate a dexamethasone responsive promoter (Fig. 1*C*).

Ecdysone Responsiveness in Stable Cell Lines. Stable cell lines were generated containing the modified ecdysone receptor VpEcR, a heterodimeric partner (RXR), and an ecdysone-inducible reporter (Fig. 2). X-Gal staining of one stable cell clone, N13, was performed. After 24 hr of treatment with 1 μM muristerone, 100% of the cells turned dark blue with 3 hr of staining (Fig. 3 *A* and *B*). Dose–response curves of stably integrated β-gal and luciferase reporters in N13 cells revealed

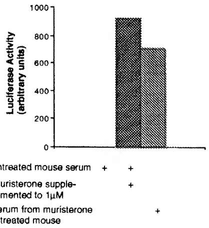

FIG. 4. Muristerone activity in mice. Adult mice were injected intraperitoneally with sesame oil with or without 10 mg of muristerone. After 12 hr, blood was drawn from the mice, and the serum was isolated. Serum from sesame oil-injected mice was divided, and half was supplemented with muristerone to a final concentration of 10 μM. The three batches of mouse serum were diluted 1:10 in DMEM and placed onto CV-1 cells transfected with CMX-GEcR, CMX-hRXRα, and EcREx5-ΔMTV-Luc.

that inducibility approaching 3 orders of magnitude can be achieved at a final concentration of 10 μM muristerone (Fig. 3*C*). One-hundred-fold induction was achieved by muristerone concentrations as low as 100 nM (Fig. 3*C*). Finally, the kinetics of muristerone-mediated induction was measured. Inductions of 100-fold in 3 hr, 1000-fold in 8 hr, and maximal effects of 20,000-fold after 20 hr of treatment were seen (Fig. 3*D*). Similar results were seen in stable lines containing CMX-VgEcR and the E/GRE reporters.

Bioavailability and Activity of Muristerone A. To use muristerone as a potential hormone in mice, its bioavailability was examined. Adult mice were injected intraperitoneally with muristerone and were subsequently sacrificed for serum collection. Serum taken 12 hr after injection was used in a transfection assay to test for muristerone activity. Serum from muristerone-

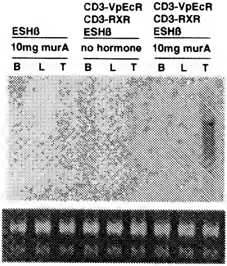

FIG. 5. Muristerone-dependent gene expression in transgenic mice. (*Upper*) Northern blot analyses were performed using 10 μg of total RNA isolated 48 hr after muristerone or sesame oil treatment. The probe used was specific to activity of the ecdysone-inducible promoter. Genotypes of the animals are described as shown. Autoradiograph was exposed for 36 hr. (*Lower*) Ethidium bromide staining of RNA prior to blotting. B, brain; L, liver; T, thymus.

treated mice yielded a luciferase activity comparable to that seen from untreated mouse serum supplemented with 1 μM muristerone (Fig. 4). Thus, not only should single-site injected material be widely circulated, but also there appears to be little or no blunting of activity due to association with serum proteins.

Muristerone-Dependent Gene Expression in Transgenic Mice. Transgenic mice were generated harboring an ecdysone-inducible reporter, ESHβ, or a T-cell-specific expression construct of VpEcR and RXR. The former will be referred to as reporter mice, the latter will be referred to as receptor mice, and double-transgenic mice will be referred to as receptor/reporter mice. Receptor mice were analyzed for VpEcR and RXR expression by Northern blot analysis of RNA collected from these mice (data not shown). These receptor mice were healthy, fertile, and by gross appearance normal. In addition, the transgene was transferred to the offspring as expected by Mendelian genetics (data not shown). This suggests that overexpression of VpEcR and RXR in T cells is not toxic. Receptor mice were then bred with reporter mice to produce double-transgenic receptor/reporter mice. After treatment with 10 mg of muristerone, RNA was isolated from various tissues including the thymus to test for specific induction of an ecdysone-inducible promoter. Muristerone treatment caused a significant induction from an ecdysone-inducible promoter, while low basal activity is observed in its absence (Fig. 5).

Comparison of Tetracycline-Based vs. Ecdysone-Inducible Systems. Transient transfection assays were performed to compare the relative activity and inducibility of the tetracycline repressor (tTA), tetracycline activator (rtTA), and modified ecdysone receptor (VgEcR/RXR) (2–4). The tetracycline repressor (tTA) activates a reporter containing tetracycline response elements (TETREs) in the absence of doxycycline, yielding a 59-fold activation (Fig. 6). This activation was completely repressed when doxycycline (0.01 μg/ml) was supplemented to the medium. In contrast, rtTA significantly activated the same reporter in the absence of doxycycline. Upon treatment with doxycycline (1 μg/ml), there was a 2.5-fold increase in activation above basal activity. This modest inducibility was due to high basal activity of rtTA, which approached maximal activity by tTA. Transfections with the ecdysone-inducible promoter (E/GRE) exhibited basal activity that was 20-fold lower than tTA and 500-fold lower than rtTA. Treatment of 1 μM muristerone A boosted reporter activity almost 1000-fold.

DISCUSSION

Tightly regulated gene expression by an exogenous inducer has numerous uses. For example, inducible expression of the cre recombinase in transgenic mice would allow for temporally specific inducible gene targeting of the adult or the developing embryo (20). Inducible expression of toxins such as the diphtheroid toxin would allow for inducible tissue-specific ablation (21).

We report here the use of the insect steroid hormone ecdysone as a potent inducer of gene activation in mammalian cells and transgenic mice. The optimized promoters containing a novel response element and the Vp16/EcR fusion receptor with an altered DNA binding specificity have yielded an extremely powerful and specific inducible system. The system's low basal activity is ideal for expression of transcription factors and toxic genes. This is likely due to the consequence that DNA binding and activation of EcR requires hormone (10). The excellent dose–response and induction rate characteristics of the ecdysone-inducible system will allow for precise control of both the amount and time period for which a desired gene is induced.

Because steroid hormones have evolved to efficiently penetrate virtually all tissues, EcR agonists like muristerone A will be useful in the study of embryonic development of any organ, including the brain. We have shown that muristerone maintains its activity when injected into mice and that it is neither toxic, teratogenic, nor inactivated by serum binding proteins. In addition to the inert qualities of muristerone, overexpression of VpEcR and RXR appears not to be toxic, at least in T cells. In transgenic mice containing an ecdysone-inducible promoter and expression of VpEcR and RXR, muristerone treatment can activate gene expression. Thus, with tissue-specific expression of VpEcR and RXR and timely hormone treatment, inducible gene expression can be achieved with spatial and temporal specificity.

In contrast to tetracycline-based systems, steroids like ecdysone offer pharmacokinetics that allow for both fast distribution and clearance of the inducer. In a direct comparison, VgEcR/RXR demonstrated both lower basal activity and higher inducibility than tTA or rtTA. Finally, in conjunction with the tetracycline-based systems, the ecdysone-inducible

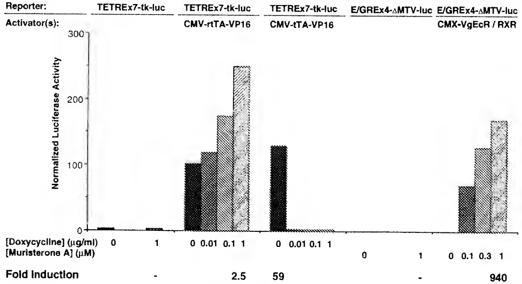

FIG. 6. Comparison of tetracycline-based and ecdysone-based inducible systems. In a transient transfection assay, CV-1 cells were transfected with equimolar amounts of TETREx7-tk-luc, E/GREx4-ΔMTV-luc, CMV-tTA-VP16, CMV-rtTA-VP16, CMX-VgEcR, and CMX-RXR, respectively. Ten times more reporter plasmid was transfected than activator(s) plasmid(s). After 24-hr hormone treatment, cells were lysed and assayed for luciferase and β-gal activities. All values have been normalized by cotransfection with CMX-β-gal.

system will allow for induction of two different genes in a temporal, spatial, and dosage-specific manner.

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